REMARKS

Claims 2 through 9 and 11 through 13 are pending in the instant application. Claims 2 through 9 and 11 through 13 have been rejected. Claims 5 and 13 have been amended. New claims 14-16 have been added. Support for these amendments is provided in the specification at page 26, lines 11-26 and Example 3. Thus, no new matter is added by these amendments. Reconsideration is respectfully requested in light of these amendments and the following remarks.

I. Objection to Disclosure

The disclosure has been objected to as the Examiner suggests that the meaning of USP 24<1211> and USP 24<71> is uncertain. The Examiner suggests that the purpose of the "<" and ">" symbols in unclear and it is uncertain as to whether USP is an abbreviation or stands for something.

The USP is a very large volume reference text updated and published annually. Use of this text and identification of protocols described therein is performed routinely by those skilled in this art field. Thus, the skilled artisan, upon reading this application would understand what is meant by the

references at page 8.

However, in an earnest effort to advance the prosecution of this case, Applicants have amended the specification to define the term USP as U.S. Pharmacopoea. Further, in accordance with the Examiner's suggestion, Applicants are providing herewith copies of pages from the U.S. Pharmacopoea for these sterility tests. These pages make clear that USP is the abbreviation used for reference to this well known text and that the protocol names are inclusive of the symbols "<" and ">".

Withdrawal of this objection to the specification is therefore respectfully requested.

II. Rejection of Claims 5-8 under 35 U.S.C. § 112, second paragraph

Claims 5-8 have been rejected under 35 U.S.C. § 112, second paragraph as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. In particular, the Examiner suggests that claim 5 is confusing and unclear for requiring a method of producing the scaffold or matrix of claim 13 without setting forth steps for a complete process to make the scaffold or

matrix. In particular, the Examiner suggests that the step of cross-linking the co-precipitate needs a step of producing the co-precipitate.

Thus, in an earnest effort to advance the prosecution, Applicants have amended claim 5 in accordance with teachings at page 16 to include a step for co-precipitation. This amendment is clearly supported by teachings in the specification and thus does not constitute new matter.

Withdrawal of this rejection under 35 U.S.C. § 112, second paragraph, is respectfully requested in light of this amendment.

III. Rejection of Claims 2-9 and 11-13 under 35 U.S.C. § 103(a)

Claims 2-9 and 11-13 have been rejected under 35 U.S.C. § 103(a) as being unpatentable over Yannas et al. (U.S. Patent 4,060,081) or Yannas et al. (U.S. Patent 4,280,954) in view of Li (U.S. Patent 5,674,290). The Examiner suggests that it would have been obvious to use electron beam radiation to carry out irradiation sterilization of the composition of Yannas et al. ('081) or Yannas et al. ('954) as suggested by Li disclosing electron beam irradiation as an alternative to gamma irradiation for sterilizing the implant made of a cross-linked co-precipitate

of collagen and glycosaminoglycan. The Examiner suggests that Li discloses a gamma irradiation dosage of 15 to 35 kGy and it would have been obvious to employ a similar dosage when using electron beam irradiation. Further, the Examiner suggests that the crosslinking conditions disclosed by Yannas et al. ('081) or ('954) would inherently provide a cross-linkage density as claimed to stabilize for electron beam radiation. In addition, the Examiner suggests that the percent glutaraldehyde in claims 6 and 8 is not unobviously different from the concentration of glutaraldehyde used by Yannas et al. ('081) and ('954).

Applicants respectfully traverse this rejection.

At the outset, Applicants respectfully disagree with the Examiner regarding the relevance of Li et al. to the instant invention. The matrices of Li et al. do not contain GAG, a required element of the matrices of the present invention. Further, Li et al. do not disclose any long-term stability of their biopolymer implants following gamma irradiation and merely provide a vague suggestion that e-beam irradiation may provide an alternative source of irradiation. Accordingly, teachings of Li et al. provide no predictability of success with respect to the instant claimed invention.

Applicants are providing herewith Declarations by Timothy

Malaney and Donald Nociolo, both of which describe experimental attempts to terminally sterilize collagen-GAG matrices.

As discussed in paragraph 3 of Timothy Malaney's Declaration, while a collagen-chondroitin-6-sulfate matrix produced by Marrion Labs which was similar in components to the instant invention performed satisfactorily in initial testing following terminal sterilization by gamma irradiation, the product did not perform adequately over time. Specifically, over time there was observed tearing of the collagen matrix, a sticky silicone bilayer and an unpleasant odor associated with the product upon opening. See paragraph 3 of Timothy Malaney's Declaration.

As discussed in paragraph 2 of Donald Nociolo's Declaration, Integra, the assignee of the instant application, has performed multiple tests on the effects of gamma irradiation doses, temperature of storage and various packaging configurations on collagen-GAG co-precipitates in an attempt to develop a stable terminally sterilized matrix. Like Marrion Labs, Integra found that initial post sterile testing of their collagen-GAG matrix terminally sterilized by gamma irradiation was acceptable. However, the product failed to meet multiple release specifications during its stability tests. See paragraph 3 of

Donald Nociolo's Declaration.

The multiple failed attempts described in Timothy Malaney's Declaration and Donald Nociolo's Declaration clearly demonstrate that the teachings of Li et al. are not predictive of success with respect to terminal sterilization of the instant claimed collagen-GAG matrix by either gamma radiation or e-beam radiation.

Further, Applicants respectfully disagree with the Examiner's suggestion that Li's disclosure of use of gamma irradiation at a dosage of 15 to 35 kGy would render it obvious to employ a similar dosage when using electron beam radiation. As discussed in paragraph 5 of Timothy Malaney's declaration, a suggestion that gamma and e-beam irradiation are interchangeable is not consistent with what is known in the art. For example, it is known that e-beam irradiation has less penetrating power than gamma irradiation. See paragraph 5 of Timothy Malaney's Declaration. Further, it is known that e-beam irradiation is more dependent upon product density, orientation, and packaging, as compared to gamma irradiation. See paragraph 5 of Timothy Malaney's Declaration.

Thus, it was only upon actual testing of e-beam terminal sterilization of the instantly claimed collagen-GAG matrix with

increased crosslinkages that Integra was able to establish that e-beam irradiation provided for a more accurate and controlled delivery of a desired dose of irradiation as compared to gamma irradiation and resulted in a sterile product that retained acceptable properties upon initial testing as well as stability testing over time. See paragraph 4 of Donald Nociolo's Declaration.

Further, Applicants respectfully disagree with the Examiner that the crosslinking conditions disclosed by Yannas et al. will inherently provide a cross-linkage density to stabilize for electron beam radiation. It is well established that the extent of glutaraldehyde crosslinking is dependent not only upon concentration but also upon conditions, i.e. pH, buffering agent, etc. The glutaraldehyde crosslinking conditions taught in Yannas are different from the glutaraldehyde conditions taught in detail in the Examples of the instant application. For example, Yannas is silent with respect to the pH of the glutaraldehyde solution while the instant application teaches glutaraldehyde in an acetic acid solution. Further, when Yannas does teach an acid, it is citric acid. Yannas also teaches soaking and storage at a pH of 7.4, while a pH of about 6.5 is taught in the instant application.

Thus, in an earnest effort to advance the prosecution of this case and to distinguish the present invention from teachings of Yannas, Applicants have amended the claims in accordance with teachings in Example 3 of the specification to clarify that the scaffold or matrix comprising a collagen and glycosaminoglycan co-precipitate of the present invention is cross-linked with glutaraldehyde at a density of cross-linkage and under conditions which stabilize the scaffold or matrix toward electron beam radiation at about 15 to about 80 kGy so that the matrix or scaffold retains characteristics to function as a structural support for cell and tissue ingrowth. Further, Applicants have added new dependent claims 14 and 15 specifying that the conditions of cross-linkage comprise glutaraldehyde in an acetic acid solution. No new matter is added by these amendments.

Further details of these conditions necessary to practice the instant claimed invention are set forth in explicit detail in the specification, for example in Example 3, and need not be set forth explicitly in the claims. See MPEP § 2164.08, *W.L. Gore & Assoc., Inc. v. Garlock, Inc.* 721 F.2d 1540, 1558, 220 USPQ 303, 316-17 (Fed. Cir. 1983) and *In re Johnson*, 558 F.2d 1008, 1017, 194 USPQ 187, 195 (CCPA 1977) which state that one does not look to the claims but to the specification to find out how to

practice the claimed invention.

Also set forth in explicit detail in the specification, for example in Examples 4 and 5, are methodologies for establishing that the density of cross-linkage as well as the conditions of cross-linkage with glutaraldehyde are adequate to stabilize the scaffold or matrix toward electron beam radiation at about 15 to about 80 kGy and provide for a matrix or scaffold which retains characteristics to function as a structural support for cell and tissue ingrowth as claimed.

Since the instant claimed invention contains limitations not taught or suggested by the combined teachings of the cited references and its success is not predicted by the combined teachings of the cited references, the instant claimed invention cannot be obvious over the combined cited references. See MPEP 2143.

Withdrawal of this rejection under 35 U.S.C. 103(a) is therefore respectfully requested.

IV. Rejection of Claims 2-8, 12 and 13 under 35 U.S.C. § 102(b)

Claims 2-8, 12 and 13 have been rejected under 35 U.S.C. 102(b) as being anticipated by Yannas et al. ('081) and ('954).

The Examiner suggests that the cross-linked collagen/ glycosaminoglycan matrix of Yannas et al. is inherently cross-linked sufficiently to retain characteristics as required by the claims.

Applicants respectfully traverse this rejection.

As discussed in detail in Section III, *supra*, the extent of glutaraldehyde crosslinking is dependent not only upon concentration but also upon conditions, i.e. pH. The glutaraldehyde crosslinking conditions taught in Yannas are different from the glutaraldehyde conditions taught in detail in the Examples of the instant application. For example, Yannas is silent with respect to the pH of the glutaraldehyde solution while the instant application teaches glutaraldehyde in an acetic acid solution. Further, when Yannas does teach use of an acid, it is citric acid. Yannas also teaches soaking and storage at a pH of 7.4, while a pH of about 6.5 is taught in the instant application.

Accordingly, in an earnest effort to advance the prosecution of this case and to distinguish the present invention from teachings of Yannas, Applicants have amended the claims to clarify that the scaffold or matrix comprising a collagen and glycosaminoglycan co-precipitate of the present invention is

cross-linked with glutaraldehyde at a density of cross-linkage and under conditions which stabilize the scaffold or matrix toward electron beam radiation at about 15 to about 80 kGy so that the matrix or scaffold retains characteristics to function as a structural support for cell and tissue ingrowth. Further, Applicants have added new dependent claims 14 and 15 specifying that the conditions of cross-linkage comprise glutaraldehyde in an acetic acid solution. Support for these amendments is provided in Example 3 of the instant application. Thus, no new matter is added by these amendments.

Since Yannas do not teach the claimed glutaraldehyde crosslinking conditions which are demonstrated in the instant application to provide for a scaffold or matrix stable toward electron beam radiation at about 15 to about 80 kGy so that the matrix or scaffold retains characteristics to function as a structural support for cell and tissue ingrowth, these references cannot anticipate the claims as amended. MPEP § 2131.

Withdrawal of this rejection under 35 U.S.C. 102(b) is therefore respectfully requested.

V. Conclusion

Applicants believe that the foregoing comprises a full and complete response to the Office Action of record. Accordingly, favorable reconsideration and subsequent allowance of the pending claims is earnestly solicited.

Respectfully submitted,

Kathleen A. Tyrrell

Kathleen A. Tyrrell
Registration No. 38,350

Date: **October 7, 2004**

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Attorney Docket No.:	**INT-0004**
Inventors:	**Mattern et al.**
Serial No.:	**10/002,653**
Filing Date:	**October 19, 2001**
Examiner:	**Naff, David M.**
Group Art Unit:	**1651**
Title:	**Collagen/Glycosaminoglycan Compositions for Use as Terminally Sterilizable Matrices**

Certificate of Facsimile Transmission

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On October , 2004

Kathleen A. Tyrrell, Registration No. 38,350

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Date of Deposit **October 7, 2004**

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By Kathleen A. Tyrrell
Typed Name: **Kathleen A. Tyrrell, Reg. No. 38,350**

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

Declaration by Donald Nociolo

I, Donald Nociolo, hereby declare:

1. I have a Bachelor of Science in Industrial Engineering from Rutgers University, and an MBA from Fairleigh Dickinson University. Prior to joining Integra LifeSciences Corporation, I worked in Engineering and Manufacturing management positions in Johnson and Johnson's Ethicon Division, and before that at Labatt's Johanna Farms Division. I am currently the Senior Vice President of Operations at Integra LifeSciences, and I've worked with Collagen/GAG matrices and scaffolds for the past 11 years at Integra LifeSciences.

2. We at Integra have performed multiple experiments examining the effects of varied gamma irradiation doses, temperature of storage, and various packaging configurations in an attempt to develop a stable matrix, which upon sterilization would retain suitability for its originally intended therapeutic use. In these experiments, the product tested was a collagen and glycosaminoglycan co-precipitate cross-linked with glutaraldehyde and packaged in normal saline.

3. While Li et al. (U.S. Patent 5,674,290) teaches that gamma irradiation can be used to sterilize an implant comprising reconstituted biopolymer which does not contain GAG, in experiments performed at Integra, we found that gamma irradiation was not an acceptable means for sterilization of our particular collagen-GAG matrix. The Integra matrix was gamma irradiated at a minimum dose of 18 kgray. The initial post sterile testing was acceptable, although there were some handling issues. However, the product failed to meet multiple release specifications during its stability tests.

4. It was only upon testing of sterilization with e-beam irradiation in combination with increased crosslinking that we found we could provide for more accurate and controlled delivery of the desired dose of irradiation as compared to gamma irradiation, resulting in a sterile product that retained acceptable properties both on initial testing and during stability testing.

I further declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such

willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Donald Nociolo

October 7, 2004
Date

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Attorney Docket No.:	**INT-0004**
Inventors:	**Mattern et al.**
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By Kathleen A. Tyrrell
Typed Name: Kathleen A. Tyrrell, Reg. No. 38,350

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

Declaration by Timothy Malaney

I, Timothy Malaney, hereby declare:

1. I have a Bachelor of Arts degree in Chemistry from the University of California, San Diego. Prior to joining Integra LifeSciences Corporation, I worked in academic and industrial positions as a protein chemist. During that time, I worked extensively with various crosslinking chemistries. As a Research Scientist, at Integra, I have worked to characterize collagen matrices. This included the characterization of different crosslinking chemistries, mechanisms of degradation,

effects of sterilization and development of new matrices based on this work.

2. The prototype Integra matrix was initially developed at Marion Labs and is composed of collagen and chondroitin-6-sulfate and used the same chemicals as the instant invention, albeit at a different concentration, to crosslink the matrices.

3. The Marrion files suggest that the initial tests of gamma sterilization of their product were satisfactory but that the product did not perform adequately over time. Among the results noted were tearing of the collagen matrix associated with a sticky silicone bilayer and an unpleasant odor associated with the product upon opening. The end product resulting from this prototype thus was prepared aseptically since attempts at terminal sterilization were unsuccessful.

4. While Li et al. (U.S. Patent 5,674,290) teaches that gamma irradiation can be used to sterilize an implant comprising reconstituted biopolymeric implant, there is no data presented in Li et al. relating to stability over time. My review of the Marrion data as well as my work at Integra indicates that testing immediately after sterilization is not predictive of product stability. Thus, data presented by Li et al. is in no way predictive of a successful product stable upon storage.

5. While both gamma and e-beam irradiation sterilize in a similar manner, e-beam irradiation has less penetrating power. It is more dependent on product density, size, orientation, and packaging. Furthermore, the variation in the magnitude of the irradiation is much greater and more difficult to control with gamma than with e-beam irradiation. To assert that gamma and e-beam irradiation are interchangeable is inconsistent with the known art.

I further declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true and further, that these statements were made with the knowledge that

willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Timothy Malaney

6 Oct 2004

Date

1

Salmonella SPECIES—By means of an inoculating loop, [...]tions from both the selenite-cystine and tetrathionate media [...] of Brilliant Green Agar Medium, Xylose–Lysine–Des-[...] Agar Medium, and Bismuth Sulfite Agar Medium con-[...] petri dishes. Cover and invert the dishes, and incubate. [...]mination, if none of the colonies conforms to the descrip-[...] *Table 4*, the specimen meets the requirements of the test [...] of the genus *Salmonella*.

[...] Morphologic Characteristics of *Salmonella* Species on Selective Agar Media

Medium	Characteristic Colonial Morphology
[...] Green [...] Medium	Small, transparent, colorless or pink to white opaque (frequently surrounded by pink to red zone)
[...]–Lysine–[...]choline Medium	Red, with or without black centers
[...] Sulfite [...] Medium	Black or green

[...] colonies of Gram-negative rods matching the description in [...] are found, proceed with further identification by transferring [...]tative suspect colonies individually, by means of an inoculat-[...] to a butt-slant tube of Triple Sugar–Iron–Agar Medium by [...]ing the surface of the slant and then stabbing the wire well [...] the surface. Incubate. If examination discloses no evidence of [...] alkaline (red) slants and acid (yellow) butts (with or [...] concomitant blackening of the butt from hydrogen sulfide [...]), the specimen meets the requirements of the test for the [...] the genus *Salmonella*.

[...] *Escherichia coli*—By means of an inoculating loop, [...] from the remaining Fluid Lactose Medium on the sur-[...] MacConkey Agar Medium. Cover and invert the dishes, and [...] Upon examination, if none of the colonies conforms to the [...] given in *Table 5* for this medium, the specimen meets the [...]ments of the test for absence of *Escherichia coli*.

Table 5. Morphologic Characteristics of *Escherichia coli* on MacConkey Agar Medium

Gram Stain	Characteristic Colonial Morphology
Negative rods (cocco-bacilli)	Brick-red; may have surrounding zone of precipitated bile

[...] colonies matching the description in *Table 5* are found, proceed [...] further identification by transferring the suspect colonies indivi-[...]ly, by means of an inoculating loop, to the surface of Levine Eo-[...] Methylene Blue Agar Medium, plated on petri dishes. If [...] colonies are to be transferred, divide the surface of each [...] into quadrants, each of which may be seeded from a separate [...]. Cover and invert the plates, and incubate. Upon examination, [...] of the colonies exhibits both a characteristic metallic sheen [...] reflected light and a blue-black appearance under transmitted [...] the specimen meets the requirements of the test for the absence [...] *Escherichia coli*. The presence of *Escherichia coli* may be con-[...] by further suitable cultural and biochemical tests.

[...]ditional, confirmatory evidence may be obtained by use of procedures set [...] in *Official Methods of Analysis of the AOAC*, 12th ed. (1975), sections [...]46.026.

Total Combined Molds and Yeasts Count—Proceed as for the *Plate Method* under *Total Aerobic Microbial Count*, except for using the same amount of Sabouraud Dextrose Agar Medium or Potato Dextrose Agar Medium, instead of Soybean Casein Digest Medium, and except for incubating the inverted petri dishes for 5 to 7 days at 20° to 25°.

Retest—For the purpose of confirming a doubtful result by any of the procedures outlined in the foregoing tests following their application to a 10.0-g specimen, a retest on a 25-g specimen of the product may be conducted. Proceed as directed under *Procedure*, but make allowance for the larger specimen size.

⟨71⟩ STERILITY TESTS

The following procedures are applicable for determining whether a Pharmacopeial article purporting to be sterile complies with the requirements set forth in the individual monograph with respect to the test for sterility. Pharmacopeial articles are to be tested by the *Membrane Filtration Method* under *Test Procedures* where the nature of the product permits. If the membrane filtration technique is unsuitable, use the *Direct Transfer Method* under *Test Procedures*. All devices, with the exception of *Devices with Pathways Labeled Sterile*, are tested using the *Direct Transfer Method*. Provisions for retesting are included under *Interpretation of Test Results*. Because sterility testing is a very exacting procedure, where asepsis of the procedure must be ensured for a correct interpretation of results, it is important that personnel be properly trained and qualified.

These Pharmacopeial procedures are not by themselves designed to ensure that a batch of product is sterile or has been sterilized. This is accomplished primarily by validation of the sterilization process or of the aseptic processing procedures.

When evidence of microbial contamination in the article is obtained by the appropriate Pharmacopeial methods, the result so obtained is conclusive evidence of failure of the article to meet the requirements of the test for sterility, even if a different result is obtained by an alternative procedure. For additional information on sterility testing, see *Sterilization and Sterility Assurance of Compendial Articles* ⟨1211⟩.

MEDIA

Prepare media for the tests as described below, or dehydrated formulations may be used provided that, when reconstituted as directed by the manufacturer or distributor, they meet the requirements of the *Growth Promotion Test*. Unless otherwise indicated elsewhere in this chapter, media are sterilized in autoclaves using a validated process.

Fluid Thioglycollate Medium

L-Cystine	0.5 g
Sodium Chloride	2.5 g
Dextrose ($C_6H_{12}O_6 \cdot H_2O$)	5.5 g
Agar, granulated (moisture content not exceeding 15%)	0.75 g
Yeast Extract (water-soluble)	5.0 g
Pancreatic Digest of Casein	15.0 g
Sodium Thioglycollate	0.5 g
or Thioglycolic Acid	0.3 mL
Resazurin Sodium Solution (1 in 1000), freshly prepared	1.0 mL
Purified Water*	1000 mL

* Distilled or deionized water can be used instead of Purified Water.

Mix and heat until solution is effected. Adjust the pH of the solution with 1 N sodium hydroxide so that after sterilization it will have a pH of 7.1 ± 0.2. Filter while hot through a filter paper, if necessary. Transfer the medium to suitable containers that provide a ratio of sur-

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face to depth of medium such that not more than the upper half of the medium has undergone a color change indicative of oxygen uptake at the end of the incubation period; and sterilize as directed above. If more than the upper one-third of the medium has a pink color, the medium may be restored once by heating the containers until the pink color disappears. When ready for use, not more than the upper one-third of the medium in a container should have a pink color. Incubate under aerobic conditions.

Alternative Thioglycollate Medium

Prepare a mixture having the same composition as that of the *Fluid Thioglycollate Medium*, but omitting the agar and the resazurin sodium solution, sterilize as directed above, and allow to cool prior to use. The pH after sterilization is 7.1 ± 0.2. Incubate under *anaerobic* conditions for the duration of the incubation period.

Soybean-Casein Digest Medium

Pancreatic Digest of Casein	17.0 g
Papaic Digest of Soybean Meal	3.0 g
Sodium Chloride	5.0 g
Dibasic Potassium Phosphate	2.5 g
Dextrose ($C_6H_{12}O_6 \cdot H_2O$)	2.5 g
Purified Water*	1000 mL

* Distilled or deionized water can be used instead of Purified Water.

Dissolve the solids in the water, heating slightly to effect a solution. Cool the solution to room temperature, and adjust the pH with 1 N sodium hydroxide so that, after sterilization, it will have a pH of 7.3 ± 0.2. Filter, if necessary, and dispense into suitable containers. Sterilize as directed above or by a validated filtration process. Incubate under aerobic conditions.

Media for Penicillins or Cephalosporins

Where sterility test media are to be used in the *Direct Transfer Method* under *Test Procedures*, modify the preparation of *Fluid Thioglycollate Medium* and *Soybean-Casein Digest Medium* as follows. To the containers of each medium, transfer aseptically a qu[...] β-lactamase sufficient to inactivate the amount of antibi[...] specimen under test. Determine the quantity of β-lactamas[...] to inactivate the antibiotic by using a β-lactamase preparati[...] been assayed previously for its penicillin- or cephalospori[...] ing power. [NOTE—Supplemented β-lactamase media ca[...] used in the membrane filtration test.]

Alternatively (in an area completely separate from tha[...] sterility testing), confirm that an appropriate amount of β-l[...] is incorporated into the medium, following either method u[...] *lidation Tests for Bacteriostasis and Fungistasis*, using less [...] colony-forming units (cfu) of *Staphylococcus aureus* (A[...] 29737) as the challenge. Typical microbial growth of the in[...] culture must be observed as a confirmation that the β-lacta[...] centration is appropriate.

Suitability Tests

STERILITY OF MEDIA

Confirm the sterility of each sterilized batch of medium by[...] ing a portion of the batch at the specified incubation tempe[...] not less than 14 days or by incubating uninoculated conta[...] negative controls during a sterility test procedure.

GROWTH PROMOTION TEST

Each lot of dehydrated medium bearing the manufacture[...] fying number or each lot of medium prepared from basic ing[...] must be tested for its growth-promoting qualities. Separately[...] late, in duplicate, containers of each medium with less than 10[...] microorganisms of each of the strains listed in Table 1, and i[...] according to the conditions specified. The test media are sati[...] if visual evidence of growth appears in all inoculated me[...] tainers within 5 days of incubation. This test can be conducted[...] taneously with the use of the media for sterility test pu[...] However, the sterility test is considered invalid if the sterility[...] media or this growth promotion test is not successful.

Table 1. Test Microorganisms[1] Suitable for Use in the *Growth Promotion Test* and the *Validation Tests for Bacteriostasis and Fun*[...]

Medium	Microorganism	Strain	Incubation (7 days) Temperature	Condition[...]
Fluid thioglycollate	*Staphylococcus aureus*[2]	ATCC 6538	32.5 ± 2.5°	aerobi[...]
	Pseudomonas aeruginosa[3]	ATCC 9027	32.5 ± 2.5°	aerobic
	Clostridium sporogenes[4]	ATCC 11437	32.5 ± 2.5°	aerobi[...]
Alternative thioglycollate[5]	*Clostridium sporogenes*	ATCC 11437	32.5 ± 2.5°	anaerob[...]
Soybean casein digest	*Bacillus subtilis*	ATCC 6633	22.5 ± 2.5°	aerobic
	Candida albicans	ATCC 10231	22.5 ± 2.5°	aerobic
	Aspergillus niger	ATCC 16404	22.5 ± 2.5°	aerobic

[1] Available from the American Type Culture Collection, 10801 University Blvd., Manassas, VA 20110-2209.
[2] An alternative to *Staphylococcus aureus* is *Bacillus subtilis* (ATCC 6633).
[3] An alternative microorganism is *Micrococcus luteus*, ATCC No. 9341.
[4] An alternative to *Clostridium sporogenes*, when a nonspore-forming microorganism is desired, is *Bacteroides vulgatus* (ATCC 8482)
[5] Use for sterility test of devices that have tubes with small lumens.

[NOTE—Seed lot culture maintenance techniques are to be used so that viable microorganisms are not more than five passages removed [...] the ATCC cultures.]

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Storage

PREPARED MEDIA—Unless otherwise indicated elsewhere in this chapter, if freshly prepared media are not used within 2 days, store them at a temperature between 2° and 25°. If prepared media are stored in unsealed containers, they can be used for one month, provided they are tested for growth promotion within two weeks of the time of use and that the color indicator requirements are met. If stored in tight containers (see *Preservation, Packaging, Storage, and Labeling* under *General Notices and Requirements*), the media can be used for one year, provided that they are tested for growth promotion within 3 months of the time of use and that the color indicator requirements are met.

READY-TO-USE MEDIA—Commercially prepared media stored in tight containers may be used provided that the requirements of the *Sterility of Media* and the *Growth Promotion Test* are met.

DILUTING AND RINSING FLUIDS FOR MEMBRANE FILTRATION

Fluid A

Preparation—Dissolve 1 g of peptic digest of animal tissue in water to make 1 liter, filter or centrifuge to clarify, if necessary, and adjust to a pH of 7.1 ± 0.2. Dispense into containers, and sterilize using a validated process.

Preparation for Penicillins or Cephalosporins—Aseptically add to the above *Preparation*, if necessary, a quantity of sterile β-lactamase sufficient to inactivate any residual antibiotic activity on the membranes after the solution of the test specimen has been filtered (see *Media for Penicillins or Cephalosporins*).

Fluid D

To each liter of *Fluid A* add 1 mL of polysorbate 80, adjust to a pH of 7.1 ± 0.2, dispense into containers, and sterilize using a validated process. Use this fluid for articles containing lecithin or oil, or for devices labeled as "sterile pathway."

Fluid K

Dissolve 5.0 g of peptic digest of animal tissue, 3.0 g of beef extract, and 10.0 g of polysorbate 80 in water to make 1 liter. Adjust the pH to obtain, after sterilization, a pH of 6.9 ± 0.2. Dispense into containers, and sterilize using a validated process.

VALIDATION TESTS FOR BACTERIOSTASIS AND FUNGISTASIS

Before instituting the use of a sterility test procedure for an article, ensure that any bacteriostatic and fungistatic activity inherent in the article to be tested does not adversely affect the reliability of the test and that the test procedure to be instituted is otherwise suitable for use with the article. Prepare dilute cultures of bacteria and fungi from the strains of microorganisms listed in Table 1 to obtain a final concentration of microorganisms in the product of less than 100 cfu per mL. Repeat the test method for each microorganism used. [NOTE—If the procedure or media specified under *Method I* does not eliminate the antimicrobial activity, alternative media or neutralizers can be used as long as they are capable of overcoming bacteriostasis or fungistasis.]

Method I

Procedure—Method I is used for validation of bacteriostasis and fungistasis by the membrane filtration method. Filter the specified quantity of the test specimen, using the same number of containers per single filter unit or canister as will be used in the sterility test. If necessary, rinse the membrane with a minimum of three 100-mL portions of the appropriate rinsing fluid. Inoculate the final rinse with less than 100 cfu. Repeat the rinse procedure on another filter that has not been exposed to the specimen under test. This filter will serve as the positive control. Place the filter or filter halves into 100-mL volumes of the specified test medium, or add the specified medium to the canister containing the membrane filter. Repeat the procedure for the appropriate microorganisms and media specified in Table 1, and incubate the containers at the appropriate temperature for not more than 7 days.

Interpretation—If the growth of each test organism in the test containers is visually comparable to the growth in the positive control, use the same amounts of article, number and volume of rinses, and medium when conducting the sterility test. If the growth of the test organisms in the test containers is not visually comparable to that in the positive control, the amount of article used is bacteriostatic or fungistatic. Repeat the test, using a larger number of rinses. Changes in the type of membrane filter used and in the use of neutralizing agents, if available, may reduce the antimicrobial effect of the article (see *Interpretation* under *Method II*). If five rinses, each of about 500 mL, fail to neutralize the antimicrobial residue on the test filter membrane, proceed with the sterility test.

Method II

Procedure—Method II is used for the validation of bacteriostasis and fungistasis by the direct transfer method. Inoculate two containers of each sterility test medium with less than 100 colony-forming units, using the volume of medium (see Table 3) for each appropriate microorganism specified in Table 1. Add the specified portion of the article under test to one of the inoculated containers of each medium. The other inoculated container is the positive control. Repeat the procedure for each appropriate microorganism, and incubate the containers at the appropriate temperature for not more than 7 days.

Interpretation—If the growth of the test organisms in the test container is not visually comparable to that of the inoculated control container, the article is bacteriostatic or fungistatic. The use of a sterile neutralizing agent, such as polysorbate 80, lecithin, azolectin, or β-lactamase, may be appropriate. If a neutralizing agent is not effective, establish suitable increased volumes of medium. Use the smallest volume of medium in which the growth of test microorganisms in the presence of the article is not adversely affected. [NOTE—If the medium volume is increased to 2000 mL and antimicrobial activity is still present, proceed with the sterility test using the 2000 mL of medium.] Volumes of medium greater than 2000 mL may be needed for testing medical devices, to permit complete immersion of the device.

GENERAL PROCEDURE

Sample Preparation

Number of Articles to Be Tested—Unless otherwise specified elsewhere in this chapter or in the individual monograph, test the number of articles specified in Table 2. If the contents of each article are of sufficient quantity (see Tables 3 and 4), they may be divided so that equal appropriate portions are added to each of the specified media. [NOTE—Perform sterility testing employing two or more of the specified media.] If each article does not contain sufficient quantities for each medium, use twice the number of articles indicated in Table 2.

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Table 2. Minimum Number of Articles to Be Tested in Relation to the Number of Articles in the Batch

Number of Articles in the Batch	Number of Articles to Be Tested
Injections / for Injections	
Not more than 100 articles	10% or 4 articles, whichever is greater
More than 100 but not more than 500 articles	10 articles
More than 500 articles	2% or 20 articles, whichever is less
For large-volume parenterals	2% or 10 containers, whichever is less
Antibiotic Solids	
—pharmacy bulk packages (< 5 g)	20 containers
—pharmacy bulk packages (≥ 5 g)	6 containers
—bulks and blends	See *Solid Bulk Products*
Products Not Intended for Injection	
Not more than 200 articles	5% or 2 articles, whichev[er is] greater
More than 200 articles	10 articles
Devices	
Not more than 100 articles	10% or 4 articles, whiche[ver is] greater
More than 100 but not more than 500 articles	10 articles
More than 500 articles	2% or 20 articles, whichev[er is] less
Solid Bulk Products	
Up to 4 containers	Each container
More than 4 but not more than 50 containers	20% or 4 containers, which[ever is] greater
More than 50 containers	2% or 10 containers, which[ever is] greater

Table 3. Quantities of Article for Liquid Products[1]

Container content (mL)	Minimum volume taken from each product container for each medium	Minimum Volume, in mL, of Each Medium: Used for direct transfer of volume taken from each container[2]	Used for membrane or half membrane representing total volume from the appropri[ate] number of containers
Less than 10	1 mL, or entire contents if less than 1 mL	15	100
10 to less than 50	5 mL	40	100
50 to less than 100	10 mL	80	100
50 to less than 100, intended for intravenous administration	½ content	200	100
100 to 500	½ contents	N/A	100
Over 500	500 mL	N/A	100
Antibiotics (liquid)	1 mL	N/A	100

[1] Constitute powder products according to the manufacturer's instructions, and then treat as liquid products.
[2] For products that cannot be tested by the membrane filtration test procedure.

Table 4. Quantities of Article for Solid Products

Container content (g)	Minimum quantity taken from each container for each medium	Minimum Volume, in mL, of Each Medium: Direct Transfer	Membrane Filtration
<50 mg	Whole content	200 mL	100 mL
>50 mg–200 mg	Half the content	200 mL	100 mL
200–300 mg	100 mg	200 mL	100 mL
300–600 mg	200 mg	200 mL	100 mL
>600 mg	200 mg	200 mL	100 mL
Antibiotic solids			
for injection (< 5 g)	150 mg	200 mL	100 mL
for injection, pharmacy bulk packages (≥ 5 g)	500 mg	200 mL	100 mL
bulks and blends	See footnote[3]	200 mL	100 mL
Surgical dressings, cotton, gauze (in packages)	100-mg portion	200 mL	N/A
Sutures and other individually packaged single-use materials	Whole devices	Not more than 2000 mL	N/A
Other medical devices	Whole devices (Cut in pieces or disassembled)	Not more than 2000 mL[2]	N/A

[1] For products that cannot be tested by the membrane filtration test procedure.
[2] Unless the device is bulky and more than 2000 mL is needed to submerge the device in the medium.
[3] See Table 2.

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...Articles—Great care must be exercised when opening an article, so that the sample to be tested for sterility is not contaminated by microorganisms present on the exterior of the container. The exterior surfaces of ampuls and closures of vials and bottles must be disinfected with a suitable decontaminating agent, and the containers should be placed in an environment that prevents recontamination of their exterior surfaces. If the vial contents are packaged under vacuum, introduce sterile air by means of a suitable sterile device, such as a needle attached to a membrane filter holder containing a sterilizing grade filter. For articles such as purified cotton, gauze, surgical dressing, suture and related Pharmacopeial articles, decontaminate the outer packaging and open the package or container aseptically.

Quantity of Article—When using the *Membrane Filtration Method*, unless otherwise specified elsewhere in this chapter or in the individual monograph, use whenever possible the entire contents of each container, but not less than the quantities specified in Table 3 and Table 4. When using the *Direct Transfer Method*, use the quantities indicated in Tables 3 and 4.

Quantity of Medium—Unless otherwise specified elsewhere in this chapter or in the individual monograph, the volume of medium used for the test is not less than the volume indicated in Table 3 or Table 4, whichever applies. [NOTE—The final volume used, however, must be adjusted according to the results of the *Validation Tests for Bacteriostasis and Fungistasis*.]

Incubation Conditions—Unless otherwise specified elsewhere in this chapter or in the individual monograph, incubate for not less than 14 days at 32.5 ± 2.5° for the *Fluid Thioglycollate Medium* or at 22.5 ± 2.5° with the *Soybean-Casein Digest Medium*, regardless of the method used for sterility testing. Observe the tubes of media on a periodic basis over the 14 days of incubation. If the test specimen is positive before 14 days of incubation, further incubation is not necessary. For products terminally sterilized by a validated moist heat process, incubate the test specimen for not less than 7 days, if the *Membrane Filtration Method* is used.

Testing Facilities

The following two types of facilities are used for sterility testing.

Clean Rooms and Clean Zones—A clean room of a sterility testing facility is maintained under microbiological control criteria appropriate for the critical zones in an aseptic processing facility (see *Microbial Evaluation of Clean Rooms and Other Controlled Environments* ⟨1116⟩). When a clean zone is used for sterility testing, it must also meet the same microbiological control criteria.

Isolators—Isolators are free-standing environments that allow aseptic manipulations to be made from outside the controlled environment. Isolator systems protect the test article and sterility test supplies from contamination during aseptic handling. Transfer ports from a dedicated autoclave or decontamination ports are used. The interior of the isolator must also meet the same microbiological control criteria.

TEST PROCEDURES

Membrane Filtration Method

APPARATUS

A suitable membrane filter unit consists of an assembly that facilitates the aseptic handling of the test articles and allows the processed membrane to be removed aseptically for transfer to appropriate media or an assembly where sterile media can be added to the sealed filter and the membrane incubated in situ. A membrane suitable for sterility testing has a rating of 0.45 µm, and a diameter of approximately 47 mm. These membranes have hydrophobic edges or low product binding characteristics that minimize inhibitory product residue, and it is this residue that interferes with the requirements of the validation test for bacteriostasis and fungistasis. For products that do not contain inhibitory substances, membranes without hydrophobic edges can be used, but wet them prior to testing. If using a sealed filter, use one that is designed to preclude product residues at the filter-unit interface. It is not necessary to use a membrane with a hydrophobic edge. The filter units and the membranes must be sterilized and stored in a manner that maintains the performance characteristics of the filter and ensures that the filter and the assembly remain sterile. When the article to be tested is an oil, the membrane and the filter assembly must be thoroughly dried before use.

SAMPLE PREPARATION

Liquids Miscible with Aqueous Vehicles—Agitate the container and aseptically transfer the specified volumes (see Table 3) of the total number of specimens tested either directly into one or more separate membrane filter units or to separate pooling vessels prior to transfer. Immediately pass each specimen through the filter with the aid of vacuum or pressure. If only one filter unit is used, aseptically remove the membrane from the holder, cut the membrane in half, and immerse half of the membrane in each of the specified media. If two or more filter units are used, place an equal number or portions of filter membrane in each of the specified media. If a closed system is used, fill an equal number of canisters with each of the specified media. [NOTE—During all manipulations, avoid excessive aeration of the *Fluid Thioglycollate Medium*.]

If the product under test has inherent bacteriostatic or fungistatic properties or contains a preservative, use *Fluid A*, and proceed as directed for *Method I* under *Validation Tests for Bacteriostasis and Fungistasis*, but exclude inoculation of the final rinse with challenge organisms.

Liquids Immiscible with Aqueous Vehicles—Proceed as directed for *Procedure* under *Liquids Miscible with Aqueous Vehicles*. If the substance under test is a viscous liquid or suspension and is not adaptable to rapid filtration, aseptically add a sufficient quantity of the appropriate diluting fluid to the pooled specimen prior to filtration to increase the flow rate.

If the substance under test contains lecithin or oil and has inherent bacteriostatic or fungistatic properties or contains a preservative, use *Fluid D*, and proceed as directed for *Method I* under *Validation Tests for Bacteriostasis and Fungistasis*, but exclude inoculation of the final rinse with challenge organisms.

Ointments and Oils Soluble in Isopropyl Myristate—Dissolve not less than 100 mg from each of 20 units (or 40 units if the contents are not sufficient for each medium) in 100 mL of isopropyl myristate that previously has been rendered sterile by filtration through a sterilizing membrane filter. [NOTE—Warm the sterile solvent, and if necessary the test material, to a maximum of 44° just prior to use.] Swirl the flask to dissolve the ointment or oil, taking care to expose a large surface of the material to the solvent. Filter this solution promptly following dissolution, keeping the filter membranes covered with the solution throughout the filtration for maximum efficiency of the filter. Wash the membranes with two 200-mL portions of *Fluid D*, then wash with 100 mL of *Fluid A*. Treat the test membranes as directed under *Liquids Miscible with Aqueous Vehicles*, except that the medium used contains 1 g of polysorbate 80 per liter.

If the substance under test contains petrolatum, use *Fluid K*, moistening the membranes with about 200 µL of the fluid before beginning the filtration. Keep the membranes covered with the prepared solution throughout the filtration operation for maximum efficiency of the filter. Following filtration of the specimen, wash the membranes with three 100-mL volumes of *Fluid K*. Treat the test membranes as directed in the previous paragraph.

Prefilled Syringes—For prefilled syringes without attached sterile needles, expel the contents of each syringe into one or two separate membrane filter funnels or into separate pooling vessels prior to transfer. If a separate sterile needle is attached, directly expel the syringe contents as indicated above, and proceed as directed for *Liquids Miscible with Aqueous Vehicles*. Test the sterility of the needle, using *Method II* under *Validation Tests for Bacteriostasis and Fungistasis*.

Solids for Injection Other than Antibiotics—Constitute the test articles as directed on the label, and proceed as directed for *Liquids Miscible with Aqueous Vehicles* or *Liquids Immiscible with Aqueous Vehicles*, whichever applies. [NOTE—If necessary, excess diluent can be added to aid in the constitution and filtration of the constituted test article.]

Antibiotic Solids for Injection—

Pharmacy Bulk Packages, < 5 g—From each of 20 containers, aseptically transfer about 300 mg of solids, into a sterile 500-mL conical flask, dissolve in about 200 mL of *Fluid A*, and mix; or constitute, as directed in the labeling, each of 20 containers and transfer a quantity of liquid or suspension, equivalent to about 300 mg of solids,

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The sterilization methods used to treat isolators, test articles, and sterility testing supplies are capable of reproducibly yielding a six-log kill against an appropriate, highly resistant biological indicator (BI; see *Biological Indicators for Sterilization* ⟨1035⟩), as verified by the fraction negative or total kill analysis methods. Total kill analysis studies are suitable for BIs with a population of 10^4 spores per unit, while fraction negative studies are suitable for BIs with a population of 10^5 or greater. A sufficient number of BIs are used to prove statistical reproducibility and adequate distribution of the sterilizing agent. Particular attention is given to areas that pose problems relative to the concentration of the agent. A larger number of BIs are used in isolators that are heavily loaded with equipment and materials. Also, when it is not possible to use one or more calibrated sensors to directly measure the concentration of the sterilizing agent, the placement of additional BIs is considered. The ability of the process to reproducibly deliver a six-log kill is confirmed in three consecutive validation studies.

The operator establishes a frequency for resterilization of the isolator. The frequency may be as short as a few days or as long as several weeks, depending on the sterility maintenance effort (see *Maintenance of Asepsis within the Isolator Environment*).

PACKAGE INTEGRITY VERIFICATION

Some materials are adversely affected by sterilizing agents, which can result in inhibition of microbial growth. Of concern are the penetration of sterilizing agents into product containers; accessory supplies such as filter sets and tubing; or any material that could come in contact with product, media, or dilution fluids used in the sterility test. It is the responsibility of the operator to verify that containers, media, and supplies are unaffected by the recommended sterilization process. Screw-capped tubes, bottles, or vials sealed with rubber stoppers and crimp overseals have proven very resistant to the penetration of commonly used sterilizing agents. Wrapping materials in metal foil or placing them in a sealed container will prevent contact with the sterilizing agent; however, these procedures may also result in some surfaces not being sterilized.

In many cases, the operator will choose to treat the surfaces of product containers under test with the sterilizing agent in order to minimize the likelihood of bioburden entering the isolator. It is the responsibility of the operator to demonstrate, via validation studies, that exposure of product containers to the sterilizing agent does not adversely affect the ability of the sterility test to detect low levels of contamination within these test articles. It is suggested that the ability of the package to resist contamination be examined using both chemical and microbiological test procedures. Bacteriostasis and fungistasis validation tests must be performed using actual test articles that have been exposed to all phases of the sterilization process (see *Sterility Tests* ⟨71⟩). This applies to medicinal device packages as well as pharmaceutical container and closure systems.

Validation studies determine whether both sterility test media and environmental control media meet the requirements for *Growth Promotion Test* under *Sterility Tests* ⟨71⟩.

MAINTENANCE OF ASEPSIS WITHIN THE ISOLATOR ENVIRONMENT

The ability of the isolator system to maintain an aseptic environment throughout the defined operational period must be validated. In addition, a microbiological monitoring program must be implemented to detect malfunctions of the isolator system or the presence of adventitious contamination within the isolator. Microbiological monitoring usually involves a routine sampling program, which may include, for instance, sampling following sterilization on the first day of operation and sampling on the last day of the projected maintenance of asepsis period. Intermediate sampling is performed to demonstrate maintenance of asepsis within the isolator.

The surfaces within the isolator can be monitored using either contact plates for flat surfaces or swabs for irregular surfaces. However, since media residues could impose a risk on isolator asepsis, these tests are generally best done at the end of the test period. If performed concurrently with testing, care is used to ensure that any residual medium is removed from isolator surfaces. Active air samples and settling plates may be used, but they may not be sufficiently sensitive to detect the very low levels of contamination present within the isolator enclosure.

The most likely route for contamination to enter the isolator is during the introduction of supplies and samples into the enclosure. Validating that all materials taken into the isolator enclosure are free of microbial contamination is critical, as is periodic inspection of gaskets to detect imperfections that could allow ingress of microorganisms. Gloves and half-suit assemblies are another likely source of microbial contamination. Gloves are of particular concern since they are used to handle both sterility testing materials and test articles. Very small leaks in gloves are difficult to detect until the glove is stretched during use. There are several commercially available glove leak detectors; the operator ensures that the detectors test the glove under conditions as close as possible to actual use conditions. Microbiological tests are used to supplement or substitute physical tests. [NOTE—Standard "finger dab plates" may not be sensitive enough to detect low levels of contamination. Submersion of the gloves in 0.1% peptone water followed by filtration of the diluent and plating on growth media can detect loss of integrity in the gloves that would otherwise go unnoticed.]

Continuous nonviable particulate monitoring within the isolator's enclosure is ideal, since it can quickly detect filter failure. A second choice is periodic monitoring using a portable particle counter. Sampling for particles must be done in a manner that poses no risk to the maintenance of asepsis within the isolator.

INTERPRETATION OF STERILITY TEST RESULTS

A sterility test resulting in a false positive in a properly functioning and validated isolator is very unlikely if bioburden is eliminated from the isolator interior with a high degree of assurance, if personnel is not in direct contact with the work area, and if the integrity of the transfer ports is validated. Nevertheless, isolators are mechanical devices and good aseptic techniques are still required. A decision to invalidate a false positive is made only after fully complying with the requirements of *Interpretation of Sterility Test Results* under *Sterility Tests* ⟨71⟩.

TRAINING AND SAFETY

As with sterility testing conducted in conventional clean rooms, operators are trained in procedures that are specific to their isolator. All training sessions and the evaluation of the operator's performance are documented in the individual's training record. Training of all personnel in the appropriate safety procedures necessary for the operation and maintenance of the isolation system is imperative.

Personnel safety in the use of a sterilizing agent must be assessed. Material Safety Data Sheets, or equivalent documents, are available in the immediate area where the sterilizing agent is being used. All storage and safety precautions are followed. An operational readiness inspection of the safety of the isolator and all associated equipment is performed and documented prior to placing the unit in service.

* ⟨1211⟩ STERILIZATION AND STERILITY ASSURANCE OF COMPENDIAL ARTICLES

This informational chapter provides a general description of the concepts and principles involved in the quality control of articles that must be sterile. Any modifications or variations in sterility test procedures from those described under *Sterility Tests* ⟨71⟩ should be validated in the context of the entire sterility assurance program and are not intended to be alternative methods to those described in that chapter.

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Within the strictest definition of sterility, a specimen would be deemed sterile only when there is complete absence of viable microorganisms from it. However, this absolute definition cannot currently be applied to an entire lot of finished compendial articles because of limitations in testing. Absolute sterility cannot be practically demonstrated without complete destruction of every finished article. The sterility of a lot purported to be sterile is therefore defined in probabilistic terms, where the likelihood of a contaminated unit or article is acceptably remote. Such a state of sterility assurance can be established only through the use of adequate sterilization cycles and subsequent aseptic processing, if any, under appropriate current good manufacturing practice, and not by reliance solely on sterility testing. The basic principles for validation and certification of a sterilizing process are enumerated as follows.

(1) Establish that the process equipment has capability of operating within the required parameters.

(2) Demonstrate that the critical control equipment and instrumentation are capable of operating within the prescribed parameters for the process equipment.

(3) Perform replicate cycles representing the required operational range of the equipment and employing actual or simulated product. Demonstrate that the processes have been carried out within the prescribed protocol limits and finally that the probability of microbial survival in the replicate processes completed is not greater than the prescribed limits.

(4) Monitor the validated process during routine operation. Periodically as needed, requalify and recertify the equipment.

(5) Complete the protocols, and document steps (1) through (4) above.

The principles and implementation of a program to validate an aseptic processing procedure are similar to the validation of a sterilization process. In aseptic processing, the components of the final dosage form are sterilized separately and the finished article is assembled in an aseptic manner.

Proper validation of the sterilization process or the aseptic process requires a high level of knowledge of the field of sterilization and clean room technology. In order to comply with currently acceptable and achievable limits in sterilization parameters, it is necessary to employ appropriate instrumentation and equipment to control the critical parameters such as temperature and time, humidity, and sterilizing gas concentration, or absorbed radiation. An important aspect of the validation program in many sterilization procedures involves the employment of biological indicators (see *Biological Indicators* ⟨1035⟩). The validated and certified process should be revalidated periodically; however, the revalidation program need not necessarily be as extensive as the original program.

A typical validation program, as outlined below, is one designed for the steam autoclave, but the principles are applicable to the other sterilization procedures discussed in this informational chapter. The program comprises several stages.

The *installation qualification* stage is intended to establish that controls and other instrumentation are properly designed and calibrated. Documentation should be on file demonstrating the quality of the required utilities such as steam, water, and air. The *operational qualification* stage is intended to confirm that the empty chamber functions within the parameters of temperature at all of the key chamber locations prescribed in the protocol. It is usually appropriate to develop heat profile records, i.e., simultaneous temperatures in the chamber employing multiple temperature-sensing devices. A typical acceptable range of temperature in the empty chamber is ±1° when the chamber temperature is not less than 121°. The *confirmatory* stage of the validation program is the actual sterilization of materials or articles. This determination requires the employment of temperature-sensing devices inserted into samples of the articles as well as *either* samples of the articles to which appropriate concentrations of suitable test microorganisms have been added, *or* separate biological indicators in operationally fully loaded autoclave configurations. The effectiveness of heat delivery or penetration into the actual articles and the time of the exposure are the two main factors that determine the lethality of the sterilization process. The *final* stage of the validation program requires the documentation of the supporting data developed in executing the program.

It is generally accepted that terminally sterilized injectable or critical devices purporting to be sterile, when processed in th clave, attain a 10^{-6} microbial survivor probability, i.e., assur less than one chance in one million that viable microorgani present in the sterilized article or dosage form. With heat-stal cles, the approach often is to considerably exceed the critic necessary to achieve the 10^{-6} microbial survivor probability kill). However, with an article where extensive heat exposu have a damaging effect, it may not be feasible to employ this c approach. In this latter instance, the development of the steril cycle depends heavily on knowledge of the microbial burden product based on examination, over a suitable time period, of stantial number of lots of the presterilized product.

The D value is the time (in minutes) required to reduce the c bial population by 90% or 1 log cycle (i.e., to a surviving frac 1/10), at a specific temperature. Therefore, where the D valu biological indicator preparation of, for example, *Bacillus s thermophilus* spores is 1.5 minutes under the total process meters, e.g., at 121°, if it is treated for 12 minutes under th conditions, it can be stated that the lethality input is 8D. The of applying this input to the product would depend on the init crobial burden. Assuming that its resistance to sterilization is e lent to that of the biological indicator, if the microbial burden product in question is 10^2 microorganisms, a lethality input yields a microbial burden of 1 (10^0 theoretical) and a furth yields a calculated microbial survivor probability of 10^{-6}. (the same conditions, a lethality input of 12D may be used in a "overkill" approach.) Generally, the survivor probability ac for the article under the validated sterilization cycle is not comp correlated with what may occur with the biological indicator. F lid use, therefore, it is essential that the resistance of the biol indicator be greater than that of the natural microbial burden article sterilized. It is then appropriate to make a worst-case as tion and treat the microbial burden as though its heat resistance equivalent to that of the biological indicator, although it is not that the most resistant of a typical microbial burden isolates w monstrate a heat resistance of the magnitude shown by this sp frequently employed as a biological indicator for steam sterili In the above example, a 12-minute cycle is considered adequ sterilization if the product had a microbial burden of 10^2 micro isms. However, if the indicator originally had 10^6 microorga content, actually a 10^{-2} probability of survival could be expe i.e., 1 in 100 biological indicators may yield positive results type of situation may be avoided by selection of the appropriat logical indicator. Alternatively, high content indicators may b on the basis of a predetermined acceptable count reduction.

The D value for the *Bacillus stearothermophilus* preparation mined or verified for these conditions should be reestablished w specific program of validation is changed. Determination of su curves (see under *Biological Indicators* ⟨1035⟩) or what has called the fractional cycle approach may be employed to dete the D value of the biological indicator preferred for the specifi ilization procedure. The fractional cycle approach may also be u evaluate the resistance of the microbial burden. Fractional cycl studied either for microbial count-reduction or for fraction ne achievement. These numbers may be used to determine the let of the process under production conditions. The data can be us qualified production equipment to establish appropriate sterili cycles. A suitable biological indicator such as the *Bacillus st thermophilus* preparation may be employed also during routin ilization. Any microbial burden method for sterility assu requires adequate surveillance of the microbial resistance of the cle to detect any changes, in addition to periodic surveillance of attributes.

Methods of Sterilization

In this informational chapter, five methods of terminal steriliza including removal of microorganisms by filtration, and guideline aseptic processing are described. Modern technological deve ments, however, have led to the use of additional procedures. T

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...de blow-molding (at high temperatures), forms of moist heat other than saturated steam and UV irradiation, as well as on-line continuous filling in aseptic processing. The choice of the appropriate process for a given dosage form or component requires a high level of knowledge of sterilization techniques and information concerning any effects of the process on the material being sterilized.[1]

STEAM STERILIZATION

The process of thermal sterilization employing saturated steam under pressure is carried out in a chamber called an autoclave. It is probably the most widely employed sterilization process.[2] The basic principle of operation is that the air in the sterilizing chamber is displaced by the saturated steam, achieved by employing vents or traps. In order to displace air more effectively from the chamber and from within articles, the sterilization cycle may include air and steam evacuation stages. The design or choice of a cycle for given products or components depends on a number of factors, including the heat lability of the material, knowledge of heat penetration into the articles, and other factors described under the validation program (see above). Apart from that description of sterilization cycle parameters, using a temperature of 121°, the F_0 concept may be appropriate. The F_0, at a particular temperature other than 121°, is the time (in minutes) required to provide the lethality equivalent to that provided at 121° for a stated time. Modern autoclaves generally operate with a control system that is significantly more responsive than the steam reduction valve of older units that have been in service for many years. In order for these older units to achieve the precision and level of control of the cycle discussed in this chapter, it may be necessary to upgrade or modify the control equipment and instrumentation on these units. This modification is warranted only if the chamber and steam jacket are intact for continued safe use and if deposits that interfere with heat distribution can be removed.

DRY-HEAT STERILIZATION

The process of thermal sterilization of Pharmacopeial articles by dry heat is usually carried out by a batch process in an oven designed expressly for that purpose. A modern oven is supplied with heated, filtered air, distributed uniformly throughout the chamber by convection or radiation and employing a blower system with devices for sensing, monitoring, and controlling the critical parameters. The validation of a dry-heat sterilization facility is carried out in a manner similar to that for a steam sterilizer described earlier. Where the unit is employed for sterilizing components such as containers intended for intravenous solutions, care should be taken to avoid accumulation of particulate matter in the chamber. A typical acceptable range in temperature in the empty chamber is ±15° when the unit is operating at not less than 250°.

In addition to the batch process described above, a continuous process is frequently employed to sterilize and depyrogenate glassware as part of an integrated continuous aseptic filling and sealing system. Heat distribution may be by convection or by direct transfer of heat from an open flame. The continuous system usually requires a much higher temperature than cited above for the batch process because of a much shorter dwell time. However, the total temperature input during the passage of the product should be equivalent to that achieved during the chamber process. The continuous process also usually necessitates a rapid cooling stage prior to the aseptic filling operation. In the qualification and validation program, in view of the short dwell time, parameters for uniformity of the temperature, and particularly the dwell time, should be established.

A microbial survival probability of 10^{-12} is considered achievable for heat-stable articles or components. An example of a biological indicator for validating and monitoring dry-heat sterilization is a preparation of *Bacillus subtilis* spores. Since dry heat is frequently employed to render glassware or containers free from pyrogens as well as viable microbes, a pyrogen challenge, where necessary, should be an integral part of the validation program, e.g., by inoculating one or more of the articles to be treated with 1000 or more USP Units of bacterial endotoxin. The test with *Limulus* lysate could be used to demonstrate that the endotoxic substance has been inactivated to not more than 1/1000 of the original amount (3 log cycle reduction). For the test to be valid, both the original amount and, after acceptable inactivation, the remaining amount of endotoxin should be measured. For additional information on the endotoxin assay, see *Bacterial Endotoxins Test* ⟨85⟩.

GAS STERILIZATION

The choice of gas sterilization as an alternative to heat is frequently made when the material to be sterilized cannot withstand the high temperatures obtained in the steam sterilization or dry-heat sterilization processes. The active agent generally employed in gaseous sterilization is ethylene oxide of acceptable sterilizing quality. Among the disadvantages of this sterilizing agent are its highly flammable nature unless mixed with suitable inert gases, its mutagenic properties, and the possibility of toxic residues in treated materials, particularly those containing chloride ions. The sterilization process is generally carried out in a pressurized chamber designed similarly to a steam autoclave but with the additional features (see below) unique to sterilizers employing this gas. Facilities employing this sterilizing agent should be designed to provide adequate post-sterilization degassing, to enable microbial survivor monitoring, and to minimize exposure of operators to the potentially harmful gas.[3]

Qualification of a sterilizing process employing ethylene oxide gas is accomplished along the lines discussed earlier. However, the program is more comprehensive than for the other sterilization procedures, since in addition to temperature, the humidity, vacuum/positive pressure, and ethylene oxide concentration also require rigid control. An important determination is to demonstrate that all critical process parameters in the chamber are adequate during the entire cycle. Since the sterilization parameters applied to the articles to be sterilized are critical variables, it is frequently advisable to precondition the load to achieve the required moisture content, to minimize the time of holding at the required temperature, prior to placement of the load in the ethylene oxide chamber. The validation process is generally made employing product inoculated with appropriate biological indicators such as spore preparations of *Bacillus subtilis*. For validation they may be used in full chamber loads of product, or simulated product. The monitoring of moisture and gas concentration requires the utilization of sophisticated instrumentation that only knowledgeable and experienced individuals can calibrate, operate, and maintain. The biological indicators may be employed also in monitoring routine runs.

As is indicated elsewhere in this chapter, the biological indicator may be employed in a fraction negative mode to establish the ultimate microbiological survivor probability in designing an ethylene oxide sterilization cycle using inoculated product or inoculated simulated product.

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[1] A number of guidelines dealing particularly with the development and validation of sterilization cycles and related topics have been published. These include, of the Parenteral Drug Association, Inc. (PDA) *Validation of Steam Sterilization Cycles* (Technical Monograph No. 1), *Validation of Aseptic Filling for Solution Drug Products* (Technical Monograph No. 2) and *Validation of Dry Heat Processes Used for Sterilization and Depyrogenation* (Technical Monograph No. 3), and of the Pharmaceutical Manufacturers Association (PMA) *Validation of Sterilization of Large-Volume Parenterals—Current Concepts* (Science and Technology Publication No. 25). Other series of technical publications on these subjects of the Health Industry Manufacturers Association (HIMA) include *Validation of Sterilization Systems* (Report No. 78-4.1), *Sterilization Cycle Development* (Report No. 78-4.2), *Industrial Sterility: Medical Device Standards and Guidelines* (Document #9, Vol. 1) and *Operator Training . . .* for *Ethylene Oxide Sterilization*, for *Steam Sterilization Equipment*, for *Dry Heat Sterilization Equipment* and for *Radiation Sterilization Equipment* Report Nos. 78-4.5 through 4.8). Recommended practice guidelines published by the Association for the Advancement of Medical Instrumentation (AAMI) include *Guideline for Industrial Ethylene Oxide Sterilization of Medical Devices—Process Design, Validation, Routine Sterilization* (No. OPEO-12/81) and *Process Control Guidelines for the Radiation Sterilization of Medical Devices* (No. RS-P 10/82). These detailed publications should be consulted for more extensive treatment of the principles and procedures described in this chapter.

[2] An autoclave cycle, where specified in the compendia for media or reagents, is a period of 15 minutes at 121°, unless otherwise indicated.

[3] See *Ethylene Oxide*, Encyclopedia of Industrial Chemical Analysis, 1971, *12*, 317-340, John Wiley & Sons, Inc., and *Use of Ethylene Oxide as a Sterilant in Medical Facilities*, NIOSH Special Occupational Hazard Review with Control Recommendations, August 1977, U. S. Department of Health and Human Services, Public Health Service, Centers for Disease Control and Prevention, National Institute for Occupational Safety and Health, Division of Criteria Documentation and Standards Development, Priorities and Research Analysis Branch, Rockville, MD.

Within ... itations of the ethylene oxide sterilization deemed s... bility of the gas to diffuse to the innermost organis... ire sterilization. Package design and chamber be app... efore must be determined so that there is minimal limit... diffusion. stra... sta... ice

STERILIZATION BY IONIZING RADIATION

...apid proliferation of medical devices unable to withstand heat ...ization and the concerns about the safety of ethylene oxide have ...lted in increasing applications of radiation sterilization. It is, how-...ver, applicable also to drug substances and final dosage forms. The advantages of sterilization by irradiation include low chemical reactivity, low measurable residues, and the fact that there are fewer variables to control. In fact, radiation sterilization is unique in that the basis of control is essentially that of the absorbed radiation dose, which can be precisely measured. Because of this characteristic, new procedures have been developed to determine the sterilizing dose. These, however, are still under review and appraisal, particularly with regard to the need, or otherwise, for additional controls and safety measures. Irradiation causes only a minimal temperature rise, but can affect certain grades and types of plastics and glass.

The two types of ionizing radiation in use are radioisotope decay (gamma radiation) and electron-beam radiation. In either case the radiation dose to yield the required degree of sterility assurance should be established such that within the range of minimum and maximum doses set, the properties of the article being sterilized are acceptable.

For gamma irradiation, the validation of a procedure includes the establishment of article materials compatibility, establishment of product loading pattern and completion of dose mapping in the sterilization container (including identification of the minimum and maximum dose zones), establishment of timer setting, and demonstration of the delivery of the required sterilization dose. For electron-beam irradiation, in addition, the on-line control of voltage, current, conveyor speed, and electron beam scan dimension must be validated.

For gamma radiation sterilization, an effective sterilizing dose which is tolerated without damaging effect should be selected. Although 2.5 megarads (Mrad) of absorbed radiation was historically selected, it is desirable and acceptable in some cases to employ lower doses for devices, drug substances, and finished dosage forms. In other cases, however, higher doses are essential. In order to validate the efficacy particularly of the lower exposure levels, it is necessary to determine the magnitude (number and/or degree) of the natural radiation resistance of the microbial population of the product. Specific product loading patterns must be established and absorbed minimum and maximum dosage distribution must be determined by use of chemical dosimeters. (These dosimeters are usually dyed plastic cylinders, slides, or squares that show color intensification based directly on the amount of absorbed radiation energy; they require careful calibration.)

The setting of the preferred absorbed dose has been carried out on the basis of pure cultures of resistant microorganisms and employing inoculated product, e.g., with spores of *Bacillus pumilus* as biological indicators. A fractional experimental cycle approach provides the data to be utilized to determine the D_{10} value of the biological indicator. This information is then applied in extrapolating the amount of absorbed radiation to establish an appropriate microbial survivor probability. The most recent procedures for gamma radiation sterilization base the dose upon the radiation resistance of the natural heterogeneous microbial burden contained on the product to be sterilized. Such procedures are currently being refined but may provide a more representative assessment of radiation resistance, especially where significant numbers of radiation-resistant organisms are present.[4] These range from inoculation with standard resistant organisms such as *Bacillus pumilus* to subprocess (sublethal) dose exposure of finished product samples taken from production lines. Certain hypotheses are common to all of these methods. While the total microbial population present on an article generally consists of a mixture of microorganisms of differing sensitivity to radiation, the step of subjecting the article to a less than totally lethal sterilization dose eliminates the less resistant microbial fraction. This results in a residual relatively homogeneous population with respect to radiation resistance, and yields consistent and reproducible results of determinations with the residual population. The amount of laboratory manipulation required is dependent upon the particular procedure used.

One such procedure requires the enumeration of the microbial population on representative samples of independently manufactured lots of the article. The resistance of the microbial population is not determined and dose setting is based on a standard arbitrary radiation resistance assigned to the microbial population, derived from data obtained from manufacturers and from the literature. The assumption is made that the distribution of resistances chosen represents a more severe challenge than the natural microbial population on the product to be sterilized. This assumption, however, is verified by experiment. After verification, the appropriate radiation sterilization dose is read from a table.

Another, more elaborate, method does not require the enumeration of the microbial population but uses a series of incremental dose exposures to allow a dose to be established such that approximately one out of 100 samples irradiated at that dose will be nonsterile. This is not the ultimate sterilization dose, but provides the basis to determine the sterilization dose by extrapolation from the dose yielding one out of 100 nonsterile samples, using an appropriate resistance factor which characterizes the remaining microorganism-resistant population. A periodic audit is conducted to check that the findings continue to be operative.

More elaborate procedures, requiring more experimentation and including the isolation of microbial cultures, include one where, after determining the substerilization dose (yielding one out of 100 nonsterile samples), the resistance of the surviving microorganisms is used to determine the sterilizing dose. Another is based on different determinations, starting with a substerilization incremental dose which results in not more than 50% of the samples being nonsterile. After irradiation of sufficient samples at this dose, a number of microbial isolates are obtained. The radiation resistance of each of these is determined. The sterilization dose is then calculated using the resistance determinations and the 50% sterilizing dose initially determined. Audit procedures are required for these methods as for the others described.

Where the required minimum radiation dose has been determined and delivery of that dose has been confirmed (by chemical or physical dosimeters), release of the article being sterilized could be effected within the overall validation of sterility assurance (which may include such confirmation of applied dosage, the use of biological indicators and other means).

STERILIZATION BY FILTRATION

Filtration through microbial retentive materials is frequently employed for the sterilization of heat-labile solutions by physical removal of the contained microorganisms. A filter assembly generally consists of a porous matrix sealed or clamped into an impermeable housing. The effectiveness of a filter medium or substrate depends upon the pore size of the porous material and may depend upon adsorption of bacteria on or in the filter matrix or upon a sieving mechanism. There is some evidence to indicate that sieving is the most important component of the mechanism. Fiber-shedding filters, particularly those containing asbestos, are to be avoided unless no alternative filtration procedures are possible. Where a fiber-shedding filter is required, it is obligatory that the process include a nonfiber-shedding filter introduced downstream or subsequent to the initial filtration step.

Filter rating—Rating the pore size of filter membranes is by a nominal rating that reflects the capability of the filter membrane to retain microorganisms of size represented by specified strains, not by determination of an average pore size and statement of distribution of sizes. Sterilizing filter membranes (those which are used for removing a majority of contaminating microorganisms) are membranes capable of retaining 100% of a culture of 10^7 microorganisms of a strain of *Pseudomonas diminuta* (ATCC 19146) per square centimeter of membrane surface under a pressure of not less than 30 psi (2 bar). Such filter membranes are nominally rated 0.22 µm or 0.2 µm, depending on the manufacturer's practice.[5] This rating of filter membranes is also specified for reagents or media that have to be st...

[4] Detailed descriptions of these procedures have been published by the Association for the Advancement of Medical Instrumentation (AAMI) in the document entitled "*Process Control Guidelines for Radiation Sterilization of Medical Devices*" (No. AAMI RS-P 10/82).

[5] Consult "Microbiological Evaluation of Filters for Sterilizing Liquids," Health Industry Manufacturers Association, Document No. 3, Vol. 4, 198...

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ized by filtration (see treatment of Isopropyl Myristate under *Ointments and Oils Soluble in Isopropyl Myristate* in the chapter *Sterility Tests* ⟨71⟩). Bacterial filter membranes (also known as analytical filter membranes), which are capable of retaining only larger microorganisms, are labeled with a nominal rating of 0.45 µm. No single authoritative method for rating 0.45-µm filters has been specified, and this rating depends on conventional practice among manufacturers; 0.45-µm filters are capable of retaining particular cultures of *Serratia marcescens* (ATCC 14756) or *Ps. diminuta*. Test pressures used vary from low (5 psi, 0.33 bar for *Serratia*, or 0.5 psi, 0.34 bar for *Ps. diminuta*) to high (50 psi, 3.4 bar). They are specified for sterility testing (see *Test Procedures Using Membrane Filtration* under the chapter *Sterility Tests* ⟨71⟩), where less exhaustive microbial retention is required. There is a small probability of testing specimens contaminated solely with small microorganisms). Filter membranes with a very low nominal rating may be tested with a culture of *Acholeplasma laidlawii* or other strain of *Mycoplasma*, at a pressure of 7 psi (0.7 bar) and be nominally rated 0.1 µm. The nominal ratings based on microbial retention properties differ when rating is done by other means, e.g., by retention of latex spheres of various diameters. It is the user's responsibility to select a filter of correct rating for the particular purpose, depending on the nature of the product to be filtered. It is generally not feasible to repeat the tests of filtration capacity in the user's establishment. Microbial challenge tests are preferably performed under a manufacturer's conditions on each lot of manufactured filter membranes.

The user must determine whether filtration parameters employed in manufacturing will significantly influence microbial retention efficiency. Some of the other important concerns in the validation of the filtration process include product compatibility, sorption of drug, preservative and/or other additives, and initial effluent endotoxin content.

Since the effectiveness of the filtration process is also influenced by the microbial burden of the solution to be filtered, the determination of the microbiological quality of solutions prior to filtration is an important aspect of the validation of the filtration process in addition to establishment of the other parameters of the filtration procedure, such as pressures, flow rates, and filter unit characteristics. Hence, another method of describing filter-retaining capability is by the log reduction value (LRV). For instance, a 0.2-µm filter that can retain 10^7 microorganisms of a specified strain will have an LRV of not less than 7, under the stated conditions.

The process of sterilization of solutions by filtration has recently achieved new levels of proficiency, largely as a result of the development and proliferation of membrane filter technology. This class of filter media lends itself to more effective standardization and quality control and also gives the user greater opportunity to confirm the characteristics or properties of the filter assembly before and after use. The fact that membrane filters are thin polymeric films offers many advantages but also some disadvantages when compared to depth filters such as porcelain or sintered material. Since much of the membrane surface is a void or open space, the properly assembled and sterilized filter offers the advantage of a high flow rate. A disadvantage is that since the membrane is usually fragile, it is essential to determine that the assembly was properly made and that the membrane was not ruptured during assembly, sterilization, or use. The housings and filter assemblies that are chosen to be used should first be validated for compatibility and integrity by the user. While it may be possible to mix assemblies and filter membranes produced by different manufacturers, the compatibility of these hybrid assemblies should first be validated. Additionally, there are other tests to be made by the manufacturer of the membrane filter, which are not usually repeated by the user. These include microbiological challenge tests. Results of these tests on each lot of manufactured filter membranes should be obtained from the manufacturer by the user for his records.

Filtration for sterilization purposes is usually carried out with assemblies having membranes of nominal pore size rating of 0.2 µm or less, based on the validated challenge of not less than 10^7 *Pseudomonas diminuta* (ATCC No. 19146) suspension per square centimeter of filter surface area. Membrane filter media which are now available include cellulose acetate, cellulose nitrate, fluorocarbonate, acrylic polymers, polycarbonate, polyester, polyvinyl chloride, vinyl, nylon, polytef, and even metal membranes, and they may be reinforced or supported by an internal fabric. A membrane filter assembly should be tested for initial integrity prior to use, provided that such test does not impair the validity of the system, and should be tested after the filtration process is completed to demonstrate that the filter assembly maintained its integrity throughout the entire filtration procedure. Typical use tests are the bubble-point test, the diffusive airflow test, the pressure hold test, and the forward flow test. These tests should be correlated with microorganism retention.

ASEPTIC PROCESSING

While there is general agreement that sterilization of the final filled container as a dosage form of final packaged device is the preferred process for assuring the minimal risk of microbial contamination in a lot, there is a substantial class of products that are not terminally sterilized but are prepared by a series of aseptic steps. These are designed to prevent the introduction of viable microorganisms into components, where sterile, or once an intermediate process has rendered the bulk product or its components free from viable microorganisms. This section provides a review of the principles involved in producing aseptically processed products with a minimal risk of microbial contamination in the finished lot of final dosage forms.

A product defined as aseptically processed is likely to consist of components that have been sterilized by one of the processes described earlier in this chapter. For example, the bulk product, if a filterable liquid, may have been sterilized by filtration. The final empty container components would probably be sterilized by heat, dry heat being employed for glass vials and an autoclave being employed for rubber closures. The areas of critical concern are the immediate microbial environment where these presterilized components are exposed during assembly to produce the finished dosage form and the aseptic filling operation.

The requirements for a properly designed, validated and maintained filling or other aseptic processing facility are mainly directed to (i) an air environment free from viable microorganisms, of a proper design to permit effective maintenance of air supply units and (ii) the provision of trained operating personnel who are adequately equipped and gowned. The desired environment may be achieved through the high level of air filtration technology now available, which contributes to the delivery of air of the requisite microbiological quality.[6] The facilities include both primary (in the vicinity of the exposed article) and secondary (where the aseptic processing is carried out) barrier systems.

For a properly designed aseptic processing facility or aseptic filling area, consideration should be given to such features as nonporous and smooth surfaces, including walls and ceilings that can be sanitized frequently; gowning rooms with adequate space for personnel and storage of sterile garments; adequate separation of preparatory rooms for personnel from final aseptic processing rooms, with the availability where necessary of such devices as airlocks and/or air showers; proper pressure differentials between rooms, the most positive pressure being in the aseptic processing rooms or areas; the employment of laminar (unidirectional) airflow in the immediate vicinity of exposed product or components, and filtered air exposure thereto, with adequate air change frequency; appropriate humidity and temperature environmental controls; and a documented sanitization program. Proper training of personnel in hygienic and gowning techniques should be undertaken so that, for example, gowns, gloves, and other body coverings substantially cover exposed skin surfaces.

Certification and validation of the aseptic process and facility are achieved by establishing the efficiency of the filtration systems, by employing microbiological environmental monitoring procedures, and by processing of sterile culture medium as simulated product.

Monitoring of the aseptic facility should include periodic environmental filter examination as well as routine particulate and microbiological environmental monitoring, and may include periodic sterile culture medium processing.

Sterility Testing of Lots

It should be recognized that the referee sterility test might not detect microbial contamination if present in only a small percentage of the finished articles in the lot because the specified number of units to

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[6] Available published standards for such controlled work areas include the following: (1) Federal Standard No. 209[illegible] Clean Room and Work Station Requirements for a Controlled Environment, Apr. 24, 1973. (2) NASA Standard for Clean Room and Work Stations for Microbially Controlled Environment, publication NHB5340.2, Aug. 1967. (3) Contamination Control of Aerospace Facilities, U. S. Air Force, T.O. 00-25-203 1 Dec. 1972, change 1-1 Oct. 1974.

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be taken imposes a significant statistical limitation on the utility of the test results. This inherent limitation, however, has to be accepted since current knowledge offers no nondestructive alternatives for ascertaining the microbiological quality of every finished article in the lot, and it is not a feasible option to increase the number of specimens significantly.

The primary means of supporting the claim that a lot of finished articles purporting to be sterile meets the specifications consist of the documentation of the actual production and sterilization record of the lot and of the additional validation records that the sterilization process possesses the capability of totally inactivating the established product microbial burden or a more resistant challenge. Further, it should be demonstrated that any processing steps involving exposed product following the sterilization procedure are performed in an aseptic manner, to prevent contamination. If data derived from the manufacturing process sterility assurance validation studies and from in-process controls are judged to provide greater assurance that the lot meets the required low probability of containing a contaminated unit (compared to sterility testing results from finished units drawn from that lot), any sterility test procedures adopted may be minimal, or dispensed with on a routine basis. However, assuming that all of the above production criteria have been met, it may still be desirable to perform sterility testing on samples of the lot of finished articles. Such sterility testing is usually carried out directly after the lot is manufactured as a final product quality control test.[7] Sterility tests employed in this way in manufacturing control should not be confused with those described under *Sterility Tests* ⟨71⟩. The procedural details may be the same with regard to media, inocula and handling of specimens, but the number of units and/or incubation time(s) selected for testing may differ. The number should be chosen relative to the purpose to be served, i.e., according to whether greater or lesser reliance is placed on sterility testing in the context of all the measures for sterility assurance in manufacture. Also, longer times of incubation would make the test more sensitive to slow-growing microorganisms. In the growth promotion tests for media, such slow growers, particularly if isolated from the product microbial burden, should be included with the other test strains. Negative or satisfactory sterility test results serve only as further support of the existing evidence concerning the quality of the lot if all of the pertinent production records of the lot are in order and the sterilizing or aseptic process is known to be effective. Unsatisfactory test results, however, in manufacturing quality control indicate a need for further action (see under *Performance, Observation, and Interpretation*).

DEFINITION OF A LOT AND SELECTION OF SPECIMENS FOR STERILITY TEST PURPOSES

Articles may be terminally sterilized either in a chamber or by a continuous process. In the chamber process, a number of articles are sterilized simultaneously under controlled conditions, for example, in a steam autoclave, so that for the purpose of sterility testing, the lot is considered to be the contents of a single chamber. In the continuous process, the articles are sterilized individually and consecutively, for example, by exposure to electron-beam radiation, so that the lot is considered to be not larger than the total number of similar items subjected to uniform sterilization for a period of not more than 24 hours.

For aseptic fills, the term "filling operation" describes a group of final containers, identical in all respects, that have been aseptically filled with the same product from the same bulk within a period of time not longer than 24 consecutive hours without an interruption or change that would affect the integrity of the filling assembly. The items tested should be representative of each filling assembly and should be selected at appropriate intervals throughout the entire filling operation. If more than three filling machines, each with either single or multiple filling stations, are used for filling a single lot, a minimum of 20 filled containers (not less than 10 per medium) should be tested for each filling machine, but the total number generally need not exceed 100 containers.

For small lots, in the case of either aseptic filling or terminal sterilization, if the number of final containers in the lot is between 20 and 200, about 10% of the containers should usually be tested. If the number of final containers in the lot is 20 or less, not fewer than 2 final containers should be tested.

Performance, Observation, and Interpretation

The facility for sterility testing should be such as to offer no greater a microbial challenge to the articles being tested than that of an aseptic processing production facility. The sterility testing procedure should be performed by individuals having a high level of aseptic technique proficiency. The test performance records of these individuals should be documented.

The extensive aseptic manipulations required to perform sterility testing may result in a probability of nonproduct-related contamination of the order of 10^{-3}, a level similar to the overall efficiency of an aseptic operation and comparable to the microbial survivor probability of aseptically processed articles. This level of probability is significantly greater than that usually attributed to a terminal sterilization process, namely, one in one million or 10^{-6} microbial survivor probability. Appropriate, known-to-be-sterile, finished articles should be employed periodically as negative controls as a check on the reliability of the test procedure. Preferably, the technicians performing the test should be unaware that they are testing negative controls. Of these tests, a false positive frequency not exceeding 2% is desirable.

For aseptically processed articles, these facts support the routine use of the test set forth under *Sterility Tests* ⟨71⟩ or a more elaborate one. The production and validation documentation should be acceptable and complete. For effectively terminally sterilized products, however, the lower microbial survivor probability may direct the use of a less extensive test than the compendial procedure specified under *Sterility Tests* ⟨71⟩, or even preclude the necessity altogether for performing one. This added reliability of sterility assurance of terminal sterilization depends upon a properly validated and documented sterilization process. Sterility testing alone is no substitute.

Interpretation of Quality Control Tests—The overall responsibility for the operation of the test unit and the interpretation of test results in relation to acceptance or rejection of a lot should be in the hands of those who have appropriate formal training in microbiology and have knowledge of industrial sterilization, aseptic processing, and the statistical concepts involved in sampling. These individuals should be knowledgeable also concerning the environmental control program in the test facility to assure that the microbiological quality of the air and critical work surfaces are consistently acceptable.

Quality control sterility tests (either according to the official referee test or modified tests) may be carried out in two separate stages in order to rule out false positive results. *First Stage.* Regardless of the sampling plan used, if no evidence of microbial growth is found, the results of the test may be taken as indicative of absence of intrinsic contamination of the lot.

If microbial growth is found, proceed to the *Second Stage* (unless the *First Stage* test can be invalidated). Evidence for invalidating a *First Stage* test in order to repeat it as a *First Stage* test may be obtained from a review of the testing environment and the relevant records thereto. Finding of microbial growth in negative controls need not be considered the sole grounds for invalidating a *First Stage* test. When proceeding to the *Second Stage*, particularly where depending on the results of the test for lot release, concurrently, initiate and document a complete review of all applicable production and control records. In this review, consideration should be paid to the following: (1) a check on monitoring records of the validated sterilization cycle applicable to the product; (2) sterility test history relating to the particular product for both finished and in-process samples, as well as sterilization records of supporting equipment, containers/closures, and sterile components, if any; and (3) environmental control data, including those obtained from media fills, exposure plates, filtering records, any sanitization records and microbial monitoring records of operators, gowns, gloves, and garbing practices.

Failing any lead from the above review, the current microbial profile of the product should be checked against the known historical profile for possible change. Records should be checked concomitantly for any changes in source of product components and/or in-processing procedures that might be contributory. Depending on the findings, and in extreme cases, consideration may have to be given to re-validation of the total manufacturing process. For the *Second Stage*, it is not possible to specify a particular number of specimens

[7] *Radioactive Pharmaceutical Products*—Because of rapid radioactive decay, it is not feasible to delay the release of some radioactive pharmaceutical products in order to complete sterility tests on them. In such cases, results of sterility tests provide only retrospective confirmatory evidence for sterility assurance, which therefore depends on the primary means thereto established in the manufacturing and validation/certification procedures.